

# 1 **Investigation on key aspects of mating biology in the mosquito *Aedes koreicus***

2 Silvia Ciocchetta<sup>\*1-2-3</sup>, Francesca D Frentiu<sup>2-4</sup>, Fabrizio Montarsi<sup>5</sup>, Gioia Capelli<sup>5</sup>, Gregor J  
3 Devine<sup>3</sup>.

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5 <sup>1</sup> School of Veterinary Science, Faculty of Science, The University of Queensland, Gatton,  
6 Australia.

7 <sup>2</sup> School of Biomedical Sciences, Queensland University of Technology, Brisbane, QLD 4000,  
8 Australia.

9 <sup>3</sup> Mosquito Control Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, QLD,  
10 Australia.

11 <sup>4</sup> Centre for Immunology and Infection Control, Queensland University of Technology,  
12 Brisbane, QLD 4000, Australia.

13 <sup>5</sup> Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, PD, Italy.

14 \* Corresponding author: [s.ciocchetta@uq.edu.au](mailto:s.ciocchetta@uq.edu.au)

## 15 **Abstract**

16 *Aedes koreicus* is a mosquito (Diptera: Culicidae) from Northeast Asia with a rapidly  
17 expanding presence outside its original native range. Over the years the species has been  
18 discovered in several new countries, either spreading after first introduction or remaining  
19 localised to limited areas. Notably, recent studies have demonstrated the ability of the species  
20 to transmit zoonotic parasites and viruses both in the field and in laboratory settings. Combined  
21 with its invasive potential, the possible role of *Ae. koreicus* in pathogen transmission highlights  
22 the public health risks of its invasion. In this study, we used a recently established population  
23 from Italy to investigate aspects of biology that influence reproductive success in *Ae. koreicus*:

24 autogeny, mating behaviour, mating disruption by the sympatric invasive species *Aedes*  
25 *albopictus*, and the presence of the endosymbiont *Wolbachia pipientis*.

26 Our laboratory population did not exhibit autogenic behaviour and required a blood meal  
27 to complete its ovarian cycle. When we exposed *Ae. koreicus* females to males of *Ae.*  
28 *albopictus*, we observed repeated attempts at insemination and an aggressive, disruptive mating  
29 behaviour initiated by males. Despite this, no sperm was identified in *Ae. koreicus*  
30 spermathecae. *Wolbachia* was not detected in this *Ae. koreicus* population and therefore had  
31 no effect on *Ae. koreicus* reproduction.

32 **Keywords:** *Aedes koreicus*, *Hulecoeteomyia koreica*, invasive mosquito species, autogeny,  
33 satyrization, *Wolbachia*.

## 34 **Introduction**

35 After its first detection in Belgium in 2008 (Versteirt *et al.*, 2012), the mosquito *Aedes*  
36 *koreicus*, commonly known as the invasive Korean bush mosquito, has invaded and established  
37 in several states in Europe and European neighbouring countries (ECDC, 2022). In some areas,  
38 such as Italy, the species is currently expanding its distribution (Gradoni *et al.*, 2021; Negri *et*  
39 *al.*, 2021; Arnoldi *et al.*, 2022), but in others, such as Germany, it has shown a relatively low  
40 tendency to spread despite suspected repeated introductions (Hohmeister *et al.*, 2021; Kurucz  
41 *et al.*, 2022).

42 While the role of *Ae. koreicus* in arthropod-borne diseases transmission is still largely  
43 unclear, the species is known to vector dog heartworm *Dirofilaria immitis* (Filarioidea:  
44 Onchocercidae) under laboratory conditions (Feng, 1930; Montarsi *et al.*, 2014), a finding later  
45 supported by field evidence of filarial DNA in *Ae. koreicus* sampled near the city of Pécs  
46 (Baranya County) in Hungary (Kurucz *et al.*, 2018). *Ae. koreicus* infection with *Wuchereria*  
47 *bancrofti* (Filarioidea: Onchocercidae) has also been documented (Yamada, 1927), and this

48 mosquito may have a role as an intermediate host for *Brugia malayi* (Filarioidea:  
49 Onchocercidae) to infect humans (KCDC, 2007). The potential of *A. koreicus* to transmit  
50 chikungunya virus was demonstrated for the first time under laboratory conditions by  
51 Ciocchetta *et al.* (Ciocchetta *et al.*, 2018). This study showed how virus transmission was  
52 temperature-dependent, and results were further confirmed by Jansen *et al.* (Jansen *et al.*,  
53 2021). The same study reported a low vector competence for Zika virus and no transmission  
54 of West Nile virus. A few studies have mentioned *Ae. koreicus*' ability to transmit Japanese  
55 encephalitis virus (JEV) in the laboratory and in the field (Miles, 1964; Gutsevich *et al.*, 1971;  
56 Takashima & Rosen, 1989). However, JEV was not detected in *Ae. koreicus* collected in Korea  
57 during more recent monitoring activities (Gutsevich *et al.*, 1970; Kim *et al.*, 2005; Kim *et al.*,  
58 2007).

59 Even though *Ae. koreicus* was first detected in Europe more than 14 years ago, its mating  
60 biology remains largely unknown. Reproductive success plays a fundamental role in mosquito  
61 establishment and population growth (Clements, 1992; Juliano & Lounibos, 2005; Takken *et*  
62 *al.*, 2006) and an assessment of the reproductive biology of *Ae. koreicus* could assist in  
63 determining its invasive potential. In this study, we investigated several important aspects that  
64 may influence the mating biology and reproductive success of the Korean bush mosquito in  
65 Italy, such as autogeny, mating behaviour and competitive mating with a sympatric invasive  
66 mosquito species (*Aedes albopictus*). We also screened the mosquito population used to derive  
67 our colony for the presence of the endosymbiont *Wolbachia pipientis*.

68 In some hematophagous arthropods, such as mosquitoes, completion of an ovarian cycle  
69 and the production of viable offspring can occur in the absence of a blood meal in a process  
70 called autogeny (Roubaud, 1929), most likely as a survival strategy when hosts are rare (Lucius  
71 *et al.*, 2017). Autogeny is hypothesised to allow the persistence of a population when the  
72 presence of vertebrate hosts is low, or to allow for rapid growth of a mosquito population at

73 the start of a season (O'Meara, 1985; Reisen & Milby, 1987). This allows mosquitoes to persist  
74 in uncertain environments and rapidly exploit optimal conditions; however, the number of eggs  
75 laid might vary considerably compared to eggs laid after a blood meal (O'Meara & Krasnick,  
76 1970; O'Meara & Edman, 1975; Mulla, 1997). Furthermore, this behaviour may delay contact  
77 with infected hosts, and could therefore impact transmission of human pathogenic viruses by  
78 mosquito vectors early in the season. Autogeny may be facultative or obligate depending on  
79 the species and environmental conditions (O'Meara & Krasnick, 1970; O'Meara & Edman,  
80 1975).

81 The autogeny phenotype has been demonstrated both in the Culicinae and Anophelinae  
82 mosquitoes (Clements, 2013). Within the Culicinae group, autogeny is commonly reported in  
83 the genus *Culex* (Provost-Javier *et al.*, 2010), and limited levels of autogeny have been  
84 observed in numerous species of *Aedes* mosquitoes (Rioux *et al.*, 1975), including some of the  
85 main mosquito threats of this century, *Ae. albopictus* and *Aedes aegypti* (Trpis, 1977;  
86 Chambers & Klowden, 1994; Mori *et al.*, 2008; Gulia-Nuss *et al.*, 2015; Aardema &  
87 Zimmerman, 2021). An essential component of autogeny is the female mating status (evidence  
88 that sperm transfer occurred): egg development in certain mosquito species does not initiate  
89 unless mating occurs, and male accessory gland products can play a central role for oogenesis  
90 (O'Meara & Evans, 1976, 1977). The ability to identify sperm in the *Ae. koreicus* female  
91 reproductive tract (mating status) is necessary to identify whether the absence of autogeny is  
92 simply the result of non-mated females. It is also fundamental in evaluating mating behaviour,  
93 reproductive success, and the subsequent spread of invasive species in a new territory.

94 The establishment of an exotic species may be hampered by the disruption of conspecific  
95 mating by the aggressive mating behaviour of males of different species (Tripet *et al.*, 2011)  
96 and by interspecific cross-insemination (satyrization) (Lounibos, 2007; Alto & Lounibos,  
97 2013). Satyrization (Ribeiro & Spielman, 1986) is a form of sterility caused by interspecific

98 mating. For example, the transfer of *Ae. albopictus* male accessory gland product to *Aedes*  
99 *aegypti* females causes them to become refractory to further mating (including with conspecific  
100 males) (Nazni *et al.*, 2009; Tripet *et al.*, 2011; Lima-Camara *et al.*, 2013). Although *Ae.*  
101 *albopictus* males are particularly efficient in satyrizing *Ae. aegypti* females, similar interactions  
102 have been noted between *Ae. albopictus* and other *Aedes* species such as *Aedes polynesiensis*  
103 and members of the *Aedes scutellaris* group (Gubler, 1970; Ali & Rozeboom, 1971a, 1971b).

104 Additionally, mosquito reproductive behaviour can be influenced by the presence of the  
105 endosymbiotic bacteria *Wolbachia pipientis*. *Wolbachia* are small (0.5–1µm), intracellular,  $\alpha$ -  
106 proteobacteria originally identified from the ovaries of *Culex* mosquitoes in 1924 (Hertig &  
107 Wolbach, 1924) and known to infect the reproductive organs of 40-60% of insect species  
108 (Jeyaprakash & Hoy, 2000; Hilgenboecker *et al.*, 2008; De Oliveira *et al.*, 2015; Weinert *et al.*,  
109 2015). They can affect host reproduction by increasing the reproductive success of infected  
110 females, thus enhancing the bacteria's maternal transmission and changing male sperm  
111 structure such that only mating with a male infected by the same bacterial strain will lead to  
112 progeny (a mechanism called cytoplasmic incompatibility) (Werren *et al.*, 2008). In some cases  
113 *Wolbachia* can induce parthenogenesis (Stouthamer *et al.*, 1999), and influence fecundity  
114 (Alexandrov *et al.*, 2007) and oogenesis (Dedeine *et al.*, 2001; Dedeine *et al.*, 2003). Our aim  
115 here was to provide the basis for further studies on the reproductive behaviour of *Ae. koreicus*  
116 and its potential to become established when introduced in new territories.

## 117 **Methods**

### 118 *Determination of autogeny in Aedes koreicus*

119 *Aedes koreicus* larvae were obtained from a colony maintained at the QIMR Berghofer  
120 Medical Research Institute (QIMRB) (Ciocchetta *et al.*, 2017). Eggs laid on Masonite® sticks  
121 were hatched in rainwater. Due to the low hatching rate of this species (Ciocchetta *et al.*, 2017),  
122 larvae were obtained from colony eggs pooled in order to produce sufficient adults for

123 experimentation. Pupae developed from larvae after nine days and were sexed using the method  
124 previously described (Ciocchetta *et al.*, 2017). To generate three experimental replicates, male  
125 and female pupae were placed together in three different cages (BugDorm<sup>®</sup> Insect Rearing  
126 Cage, 30 x 30 x 30 cm) at the following initial numbers: cage 1, 161 males - 163 females; cage  
127 2, 161 males - 174 females; cage 3, 161 males - 170 females.

128 The cages of adults were maintained in environmental chambers (Panasonic, Osaka,  
129 Japan) as described previously (Ciocchetta *et al.*, 2017). A 10% w/v sucrose solution was  
130 provided *ad libitum* and each cage was equipped with one egg collection tray (© 2014 Genfac  
131 Plastics Pty Ltd, 18.3 x 15.2 x 6.5 cm) with rainwater and Masonite<sup>®</sup> sticks as oviposition  
132 substrates (Figure 1). The position of the cages within the environmental chamber was changed  
133 twice per week to minimise positional bias. The number of emerging adults was counted, and  
134 cages were checked daily for eggs. After three weeks of caging, one of the three cages was  
135 randomly chosen (cage 2) to proceed to blood feeding on human volunteers (QIMRB Human  
136 Research Ethics Committee approval HREC361). The percentage of fed mosquitoes was  
137 recorded. Two weeks after blood feeding (and seven days from the start of oviposition), eggs  
138 were collected, counted, and stored in an anti-leak plastic bag. Additionally, 5 female  
139 mosquitoes from the blood-fed cage and 10 female mosquitoes from the remaining two cages  
140 were killed (using CO<sub>2</sub>), and their ovaries were dissected in a drop of phosphate-buffered saline  
141 (PBS) on a glass slide at a magnification of 10x in order to identify mature follicles (stage IVb  
142 and V) (Christophers, 1911; Clements & Boocock, 1984; Armbruster & Hutchinson, 2002;  
143 Hugo *et al.*, 2003; Itina *et al.*, 2014). The viability of a subsample of eggs collected from cage  
144 2 (n= 1189) was measured after 14 days of storage (Ciocchetta *et al.*, 2017) to verify the  
145 successful completion of the gonotrophic cycle in that cage. Observation of Masonite<sup>®</sup> sticks  
146 for presence of eggs in the non-blood-fed cages continued until all adult mosquitoes had died  
147 and the absence of autogeny was confirmed.



148

149 **Figure 1** Egg collection tray with rainwater and Masonite<sup>®</sup> sticks.

150 *Conspecific Aedes koreicus mating behaviour*

151 *Aedes koreicus* pupae were derived from mosquito eggs laid on Masonite<sup>®</sup> sticks and  
152 sexed according to Ciocchetta et al. (Ciocchetta *et al.*, 2017). 190 males and 240 females were  
153 separated into two different BugDorm<sup>®</sup> cages placed in environmental chambers for  
154 emergence, at the previously described colony rearing temperature and relative humidity  
155 (Ciocchetta *et al.*, 2017). Preliminary observations demonstrated that *Ae. koreicus* mosquitoes  
156 mate under conditions of scarce illumination (Silvia Ciocchetta, personal observation). As a  
157 result, the light/dark cycle was reversed so that mosquito behaviour could be observed under  
158 crepuscular and dark conditions. The observation cage was a modified BugDorm<sup>®</sup> cage with  
159 transparent plexiglass used on one side of the cage instead of mesh. Male mosquitoes require  
160 a sufficient period of time for genitalia and sperm development before mating (Oliva *et al.*,  
161 2014b), whereas females are often receptive as soon as they emerge (Takken *et al.*, 2006). As  
162 a result, 6-7-day old virgin males and 2-3-day old virgin females were caged together, and their  
163 behaviour recorded. At 12-13 hours intervals, 25 females were aspirated from the experiment  
164 cage, anaesthetised with CO<sub>2</sub> and dissected in a drop of phosphate-buffered saline (PBS) on a  
165 glass slide at 10x magnification. A cover slip was used to rupture the spermathecae and allow  
166 for sperm visualisation at an increased magnification of 40x.

167 *Preliminary observations of Aedes albopictus and Aedes koreicus mating disruption*

168 *Aedes koreicus* larvae were reared as previously described (Ciocchetta *et al.*, 2017).  
169 *Aedes albopictus* larvae (from a colony established at QIMR from eggs collected on Hammond  
170 Island, Torres Strait, Australia, in May 2014) were similarly reared, but at a temperature of 27  
171  $\pm 1^\circ\text{C}$ . The colonies of both species were synchronised to pupate at the same time. Pupae were  
172 individually placed in Falcon<sup>®</sup> tubes containing 5 to 10 ml of rainwater to allow the collection  
173 of emerging virgin males or females. 3-4 days old *Ae. albopictus* males (N=27) ready for copula  
174 (Oliva *et al.*, 2014b) and 2-3 days old virgin *Ae. koreicus* females (N=22) were introduced in  
175 a BugDorm<sup>®</sup> cage containing a solution of 10% w/v sucrose. The interaction between the two  
176 mosquito species was recorded utilising a GoPro<sup>®</sup> Hero 3 camera. After five days, all female  
177 mosquitoes were anaesthetised with CO<sub>2</sub> and the spermathecae were dissected in a drop of saline  
178 buffer, crushed under a cover slip and scanned at 40x magnification for the presence of sperm.

179 *Wolbachia presence in field-collected Aedes koreicus*

180 Field-collected *Aedes koreicus* sampled during a survey carried out in north-eastern Italy  
181 from 2011 to 2015 (Montarsi *et al.*, 2015), from the same population used to derive our QIMRB  
182 colony (Ciocchetta *et al.*, 2017), were screened for the presence of *Wolbachia pipientis*.  
183 Females (n=21) collected in Belluno (46°08'44.3"N 12°12'38.0"E) in July 2014, were  
184 preserved in RNALater<sup>®</sup> (Invitrogen<sup>™</sup>), and stored at  $-80^\circ\text{C}$ . DNA was extracted using  
185 QIAGEN DNeasy<sup>®</sup> Blood and Tissue Kit. The extracted DNA was utilised as a template for  
186 the polymerase chain reaction (PCR) targeting the *Wolbachia*-specific *wsp* and *16s* genes and  
187 the mosquito housekeeping *RpS17* gene, which acted as a positive control for the extraction:

188 (*wsp* F: 5'– TGGTCCAATAAGTGATGAAGAAAC–3', R: 5'–

189 AAAAATTAAACGCTACTCCA–3'; *16s* F: 5'–TTGTAGCCTGCTATGGTATAACT–3',

190 R: 5'– GAATAGGTATGATTTTCATGT–3'; *RpS 17* F: 5'–



191 TCCGTGGTATCTCCATCAAGCT-3', R: 5'-CACTTCCGGCACGTAGTTGTC-3')

192 (O'Neill *et al.*, 1992; Braig *et al.*, 1998; Cook *et al.*, 2006).

193 PCR with *wps* primers was performed using a Phusion® High-Fidelity PCR Kit with  
194 initial denaturation at 98°C for 30 sec, followed by a 34 cycles consisting of 98°C for 10  
195 seconds, 59°C for 30 seconds, and 72°C for 30 seconds and a final extension step at 72°C for  
196 10 minutes. The same protocol was applied with *16s* and *RpS17* primers, but the annealing  
197 temperatures were 56°C for *16s* primers and 58°C for *RpS17* primers.

198 DNA for four *Wolbachia*-positive controls was extracted from *wMel*-infected *A. aegypti*  
199 maintained in the QIMR Berghofer insectary (Ulrich *et al.*, 2016) using the same extraction kit  
200 of the target samples. In each PCR, a sample from an *Ae. aegypti* wildtype colony (QIMRB)  
201 that was negative for *Wolbachia* was also tested. DNA from *Culex sitiens* mosquitoes (n=3)  
202 infected with *Wolbachia* (QIMRB colony) was extracted using QuickExtract™ DNA  
203 Extraction Solution (Epicentre Technologies Corporation) and tested as an additional positive  
204 control.

## 205 **Results**

### 206 *Determination of autogeny in Aedes koreicus*

207 Proportion of male: female totals were 123:134, and 103:138 in the two non-blood fed  
208 cages (cages 1 and 3), and 116:146 in the 3<sup>rd</sup>, blood fed cage (cage 2). No eggs were observed  
209 in the oviposition trays of the three cages for 21 days after co-caging. After this period, a  
210 volunteer fed the mosquitoes in the cage designated to be blood fed (97.2% fed, n= 109) and  
211 oviposition on the Masonite® sticks in that cage occurred seven days post-feeding. Mature  
212 follicles were observed in all 5 mosquitoes dissected from that cage. No eggs were observed  
213 on Masonite® sticks in cages 1 and 3, and no mature follicles were found in the female  
214 mosquitoes dissected from those cages. The percentage of male and female mosquitoes still  
215 alive at the time of these observations (28 days after co-caging) were: cage 1= males 8.9% (n

216 = 123), females 59.7% (n = 134); cage 2 = males 44.8% (n = 116), females 67.1% (n = 146);  
217 cage 3 = males 25.2% (n = 103), females 71.0% (n = 138). A total of 4,925 eggs were counted  
218 under the stereoscope from the Masonite® sticks collected from the blood fed cage; the average  
219 eggs/female = 50.25 was consistent with a previously reported fecundity index (Ciocchetta *et*  
220 *al.*, 2017).

### 221 *Conspecific Aedes koreicus* mating behaviour

222 No sperm was observed in spermathecae from female mosquitoes dissected  $12 \pm 0.5$  and  
223  $25 \pm 0.5$  hours after co-caging. Mating activity was observed after 25.5 hours showing *Aedes*  
224 *koreicus* males and females in the act of copula and documented with a smartphone device  
225 (Oppo F1 Android smartphone; supplemental file 1: Ae. koreicus mating.mp4). Evidence of  
226 motile sperm in *Ae. koreicus* female spermathecae (Figure 2) was found in 28% of females  
227 (n=25) sampled 31 hours after co-caging with males (females were sampled approximately five  
228 hours after evidence of mating activity in the cage to allow the sperm a sufficient period to  
229 reach the spermathecae (Oliva *et al.*, 2014b).

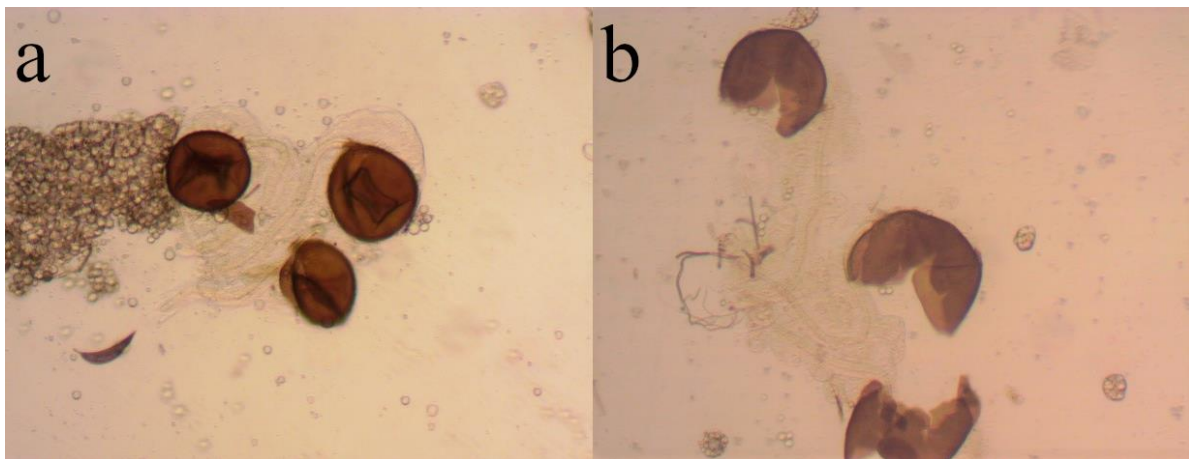


230

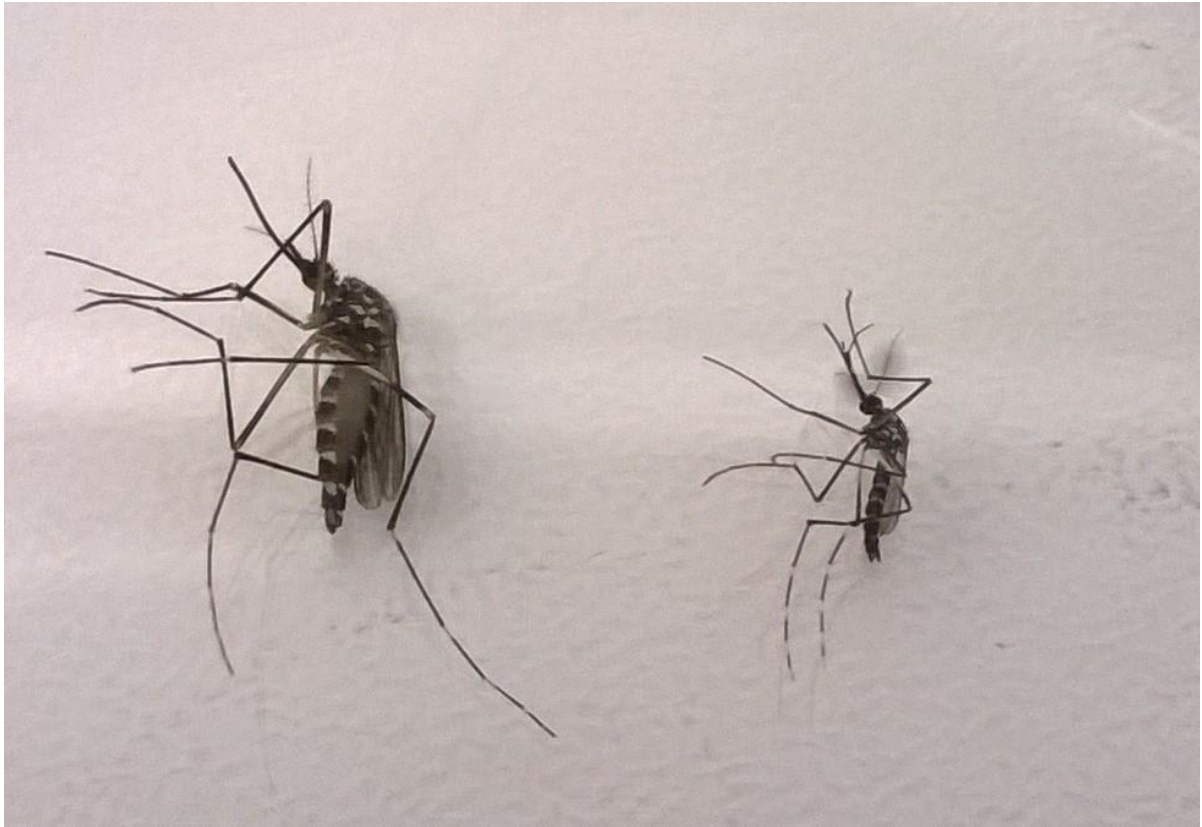
231 **Figure 2** *Ae. koreicus* sperm visible after spermathecae rupture.

232 *Preliminary observations on Aedes albopictus and Aedes koreicus mating disruption*

233 Despite repeated interactions between *Aedes koreicus* females and *Aedes albopictus*  
234 males (supplemental file 2: Ae. albopictus\_Ae.koreicus interaction.mp4), no sperm was  
235 detected in the 22 individuals dissected (Figure 3). Differences in the size of the mosquitoes  
236 (*Ae. koreicus* females were visibly bigger than *Ae. albopictus* males; Figure 4) may have been  
237 a possible cause for the failure in interspecific insemination. Although the specific size of each  
238 individual was not measured, *Ae. koreicus* females wing length for individuals reared according  
239 to previous work (Ciocchetta et al., 2017) has been reported to be over 3 mm; in their work  
240 Pudar et al. (Pudar et al., 2021) reported an *Ae. albopictus* male wing length of approximately  
241 2mm when the species was reared at a temperature close to our experiment ( $28 \pm 1$  °C). When  
242 reared at the same rearing conditions, these two species maintained their differences in  
243 dimensions, with *Ae. koreicus* bigger in size when compared to *Ae. albopictus* (Baldacchino et  
244 al., 2017), supporting our observations.



245  
246 **Figure 3** No evidence of *Ae. albopictus* sperm in *Ae. koreicus* spermathecae (a) before and (b)  
247 after rupture.

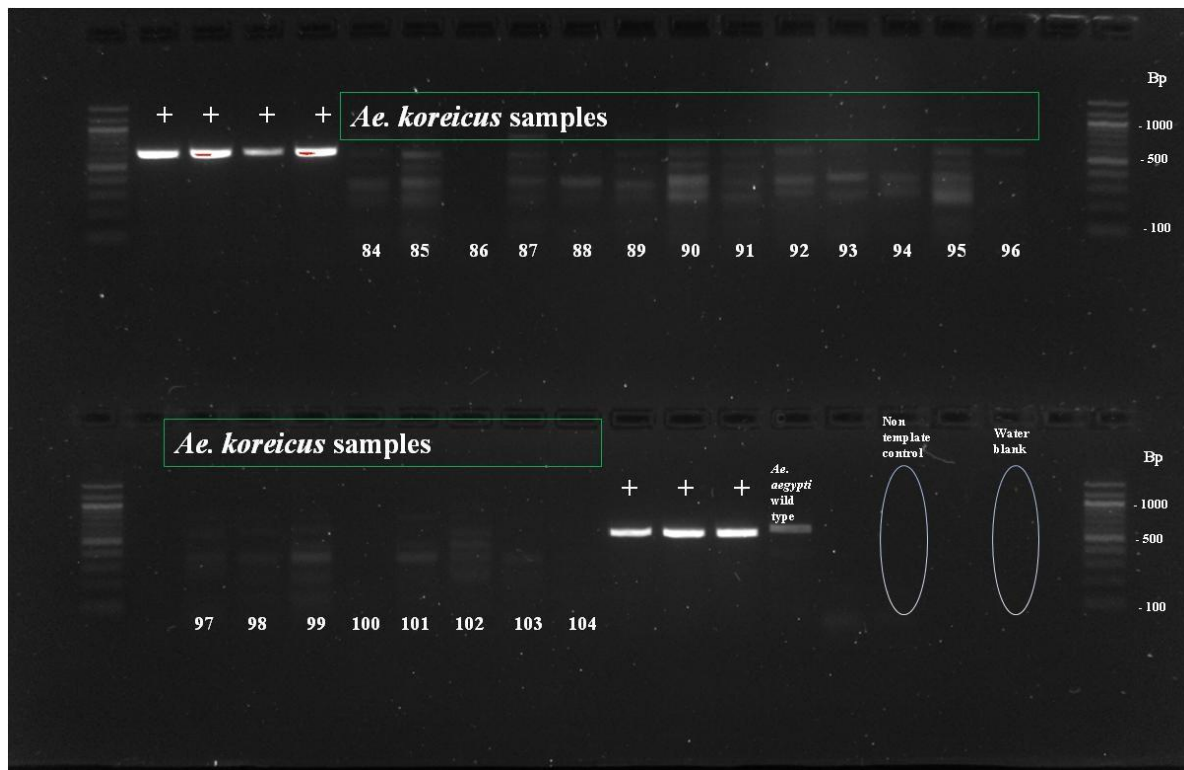


248

249 **Figure 4** Difference in size between *Ae. koreicus* female (left) and *Ae. albopictus* male (right).

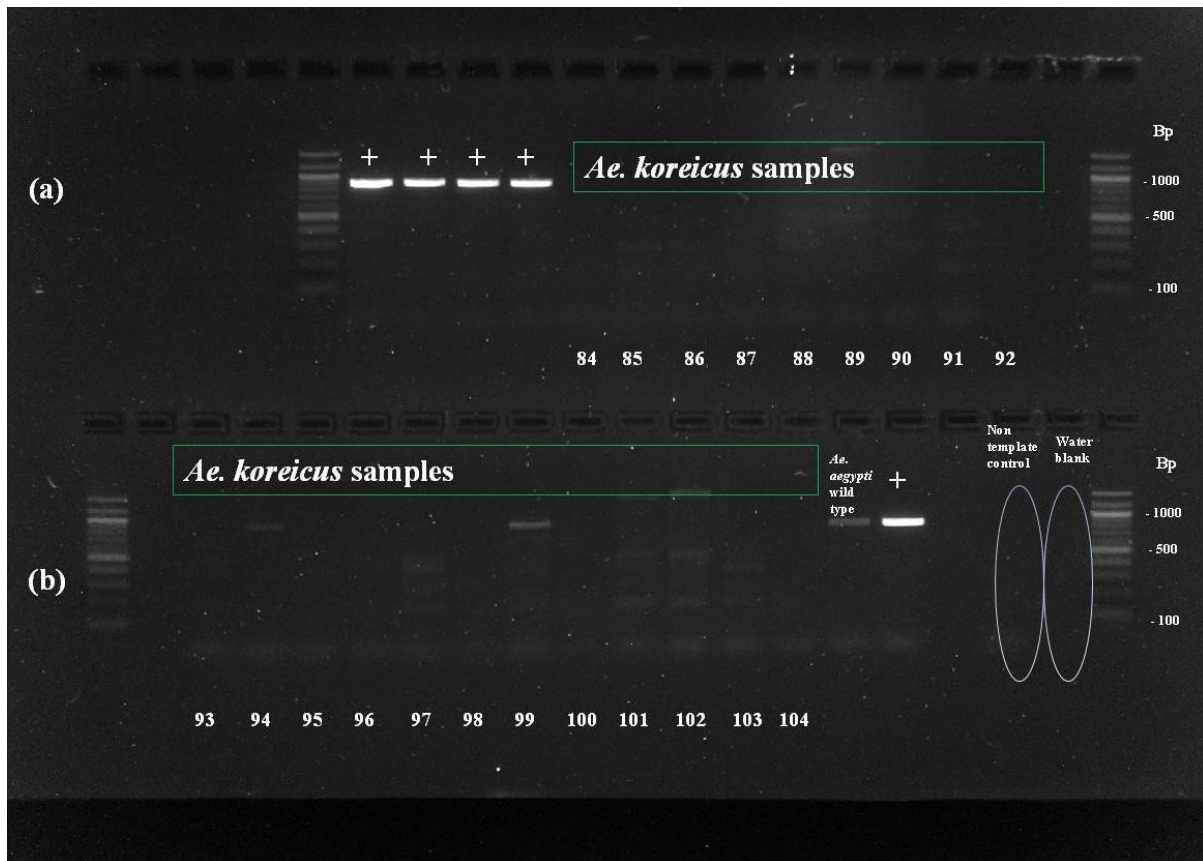
250 *Wolbachia* presence in field-collected *Ae. koreicus*

251 No *Wolbachia* was identified in the *Aedes koreicus* field samples. The DNA extraction  
252 was validated by running a PCR analysis using *RpS17* housekeeping gene primers for mosquito  
253 DNA (Figure 5-7), and *Wolbachia* was detected by the *wsp* and *16S* primers in all positive  
254 controls.



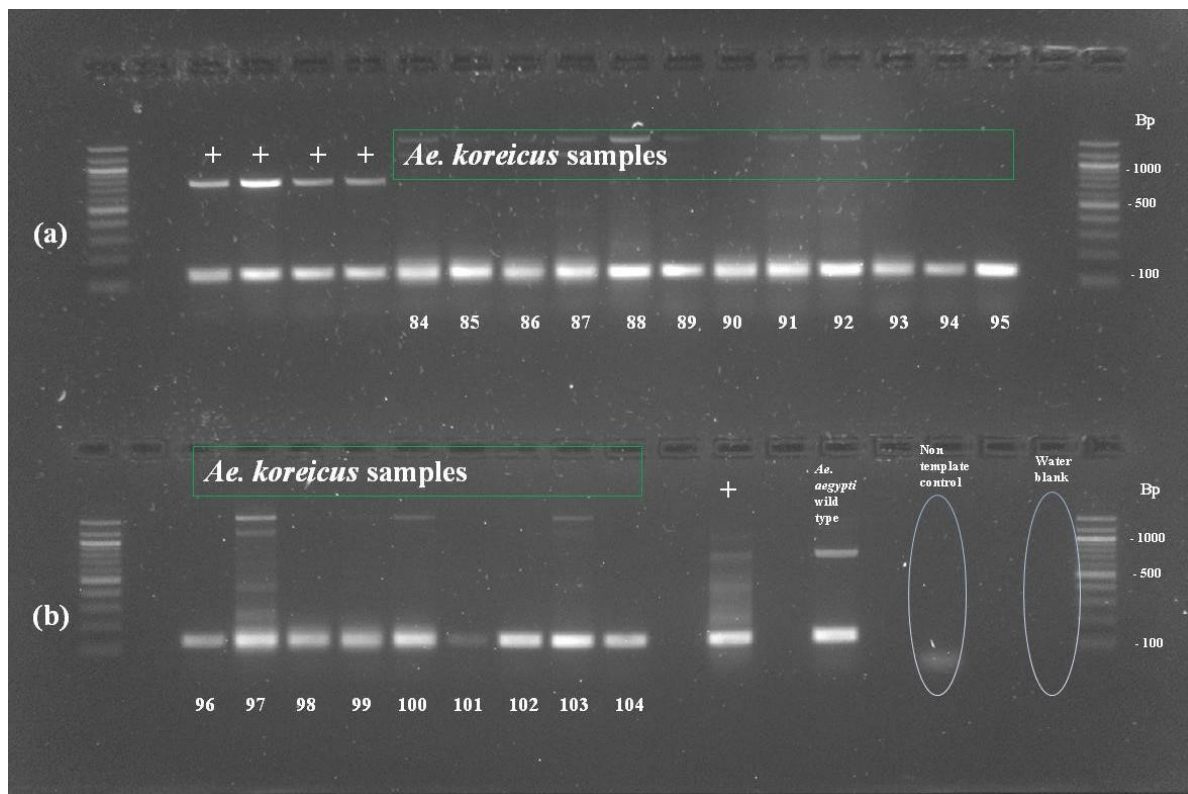
255

256 **Figure 5** PCR products produced by amplifying *Ae. koreicus* DNA with oligonucleotide  
257 primers corresponding to *Wolbachia* gene *wsp*. (a) positive controls indicated by the symbol +  
258 (*Ae. aegypti* *Wolbachia* infected, QIMR Berghofer), *Ae. koreicus* samples lanes 84 to 96; (b)  
259 *Ae. koreicus* lanes 97 to 104, positive controls indicated by the symbol + (*Culex sitiens*  
260 *Wolbachia* infected, Chen Wu, QIMR Berghofer), *Ae. aegypti* wildtype *Wolbachia*-free, QIMR  
261 Berghofer.



262

263 **Figure 6** PCR products produced by amplifying *Ae. koreicus* DNA with oligonucleotide  
264 primers corresponding to *Wolbachia* gene *16S*. (a) positive controls indicated by the symbol +  
265 (*Ae. aegypti* *Wolbachia* infected, QIMRB), *Ae. koreicus* lanes 84 to 92; (b) *Ae. koreicus* lanes  
266 93 to 104, positive controls indicated by the symbol + (*Culex sitiens* *Wolbachia* infected, Chen  
267 Wu, QIMRB), *Ae. aegypti* wildtype *Wolbachia*-free, QIMRB.



268

269 **Figure 7** PCR products produced by amplifying *Ae. koreicus* DNA with oligonucleotide  
270 primers corresponding to the housekeeping gene *RsPI7*. (a) positive controls indicated by the  
271 symbol + (*Ae. aegypti* *Wolbachia* infected, QIMR Berghofer), *Ae. koreicus* lanes 84 to 95; (b)  
272 *Ae. koreicus* lanes 96 to 104, positive controls indicated by the symbol + (*Culex sitiens*  
273 *Wolbachia* infected, Chen Wu, QIMR Berghofer), *Ae. aegypti* wildtype *Wolbachia*-free, QIMR  
274 Berghofer.

## 275 Discussion

276 Defined as the ability to produce offspring in the absence of a blood meal, autogeny can  
277 influence the vector potential of a mosquito by affecting the abundance or persistence of  
278 vectors, even in the absence of immediate hosts (Reisen & Milby, 1987; Tsuji *et al.*, 1990).  
279 Conversely, autogeny may limit contact with hosts and reduce transmission risks (Spadoni *et*  
280 *al.*, 1974; Reisen & Milby, 1987). Our results suggest that *Ae. koreicus* mosquitoes do not  
281 display this phenotype under the conditions of our experiment. There was no oviposition when

282 mosquitoes were deprived of a blood source. In early studies with the mosquito *Aedes*  
283 *taeniorhynchus*, O'Meara *et al.* (O'Meara & Evans, 1976) showed that mating may increase  
284 the levels of autogeny and that the expression of autogeny is correlated to the environmental  
285 conditions in which the larval stages develop and the geographical origin of the population  
286 (O'Meara, 1979). In *Ae. taeniorhynchus*, mating was necessary only when larvae were exposed  
287 to conditions unfavourable to their development and was otherwise not required for the  
288 production of viable eggs (O'Meara, 1979). The observation of *Ae. koreicus* mating behaviour  
289 and the detection of sperm in *Ae. koreicus* spermathecae confirmed that the absence of  
290 autogeny was not due to a lack of mosquito mating. Moreover, autogenic populations of *Ae.*  
291 *japonicus*, a species phylogenetically close to *Ae. koreicus*, have never been reported in the  
292 literature. We hypothesised that *Ae. koreicus* may be an anautogenous mosquito species;  
293 however, although autogeny was not present in the studied colony, the phenotype could still be  
294 present in different *Ae. koreicus* populations, as previously found for instance in *Ae. albopictus*  
295 (Chambers & Klowden, 1994; Mori *et al.*, 2008).

296         The delay of 25.5 hours being observed before mosquito mating could be due to different  
297 factors. Although adult female mosquitoes are ready to be inseminated once they emerge, male  
298 antennae and genitalia at the moment of imaginal stage emergence are not in the correct  
299 morphological conformation to allow copula. Physical changes must occur for the males to  
300 become sexually active (Oliva *et al.*, 2014a). These changes include the erection of fibrillar  
301 hairs in the antennae, (important for female localisation (Roth, 1948)), and the permanent 180°  
302 rotation of terminalia part of the genitalia to correctly orient the male genital structure for  
303 mating (Lamb, 1922). In particular, the time required for this rotation varies among mosquito  
304 species and can take up to four days, for example, as reported in the species *Aedes provocans*  
305 (Smith & Gadawski, 1994). The time of *Ae. koreicus* male genitalia rotation is not known,  
306 which justifies the choice to cage females with 6-7 days old virgin males. Moreover, mating



307 may be encouraged by behaviours displayed in the wild, such as swarming (Cabrera & Jaffe,  
308 2007), that are challenging to create in a laboratory colony.

309 In this preliminary exploration of *Ae. koreicus* and *Ae. albopictus* mating interactions,  
310 *Ae. albopictus* males showed repeated and aggressive mating attempts towards *Ae. koreicus*  
311 but were unable to transfer sperm to *Ae. koreicus*. The different sizes of the two species might  
312 be one explanation for how this has played a role in the outcome of this experiment, with the  
313 wing length for females of *Ae. koreicus* being reported over 3mm and *Ae. albopictus* male wing  
314 length approximately 2mm (Baldacchino *et al.*, 2017; Ciocchetta *et al.*, 2017; Pudar *et al.*,  
315 2021). Yet, the lack of sperm doesn't necessarily exclude a satyrization effect produced by *Ae.*  
316 *albopictus* males, because the transfer of male accessory glands products (responsible for the  
317 satyrization effect) may occur even in the absence of sperm in the spermathecae, as  
318 demonstrated by Carrasquilla and Lounibos (Carrasquilla & Lounibos, 2015). Although  
319 satyrization between these two species seems unlikely, the aggressive mating attempts shown  
320 by *Ae. albopictus* males towards *Ae. koreicus* females could prevent less aggressive *Ae.*  
321 *koreicus* males from mating, and therefore lead to a decrease in *Ae. koreicus* numbers in the  
322 field.

323 Samples tested for *Wolbachia* were collected during the early stages of *Ae. koreicus*  
324 invasion in Italy. *Wolbachia* was not detected in *Ae. koreicus* from the first established field  
325 population in Belluno from which the studied colony was derived. Therefore, the bacterial  
326 endosymbiont is unlikely to have affected *Ae. koreicus* reproductive behaviour in the initial  
327 establishment of this mosquito in Italy. The absence of *Wolbachia* in *Ae. koreicus* has been  
328 confirmed by subsequent studies in immature and adult stages sampled in the Province of  
329 Trento in 2015 and 2017 (Rosso *et al.*, 2018; Alfano *et al.*, 2019). It should be noted that in our  
330 preliminary investigation we tested a small sample (N=21) of *Ae. koreicus* females;  
331 nonetheless, our results suggest that even if present in the mosquito population initially

332 established in Italy, the prevalence of *Wolbachia* was low. Interestingly, a more recent study  
333 (Damiani *et al.*, 2022) on samples collected between 2019 and 2020 in several villages in  
334 North-East Italy detected *Wolbachia* in 2 of the 85 females examined. Further research with  
335 larger sample sizes could help establish whether *Wolbachia* is present at a very low prevalence  
336 in the *Ae. koreicus* population established in Italy or whether the recent discovery is due to  
337 introgression (Bargielowski *et al.*, 2015) from other mosquito species carrying the  
338 endosymbiont (*Wolbachia* strains isolated in *Ae. koreicus* are closely related to the *wAlbB*  
339 strain, one of the two native strains of *Ae. albopictus* (McMeniman *et al.*, 2009). In the Province  
340 of Trento for instance, *Ae. albopictus* had 2.5% prevalence of *Wolbachia* (Rosso *et al.*, 2018).

341       Considering the importance played by reproductive success in ensuring the establishment  
342 and growth of invasive mosquito populations during the colonization of new territories, our  
343 preliminary results are aimed at informing further studies to assist in determining the invasive  
344 potential of *Ae. koreicus* and the public health risk posed in areas of recent introduction.

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#### 348 **Conflict of Interest**

349 The authors declare that they have no competing interests.

#### 350 **Author Contributions**

351 Conceived and designed the study: SC, FDF and GJD. Collected the data: SC. Analysed the  
352 data: SC. Drafted the manuscript: SC. Reviewed the manuscript: FDF, FM, GC and GJD. All  
353 authors read and approved the final manuscript.

354

355 **Data Availability**

356 The data supporting the conclusions of this article are included within the article and its  
357 additional files.

358 **Supporting Information**

359 **Ae. koreicus mating.mp4** mp4 video, 7.92 MB *Ae. koreicus* males and females in the act  
360 of copula

361 **Ae. albopictus\_Ae.koreicus interaction.mp4** mp4 video, 3.07 MB Repeated

362 interactions between *Ae. koreicus* female and *Ae. albopictus* males

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